The 9-Arginine Residue of α-Conotoxin GI Is Responsible for Its Selective High Affinity for the αγ Agonist Site on the Electric Organ Acetylcholine Receptor†

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Received April 8, 1997; Revised Manuscript Received May 19, 1997⊗

ABSTRACT: The two agonist-binding domains of the electric organ nicotinic acetylcholine receptor are located at the αγ and αδ subunit interfaces. α-Conotoxins GI and MI are competitive antagonists of this receptor and, like *d*-tubocurarine, bind to the αγ site with much higher affinity than to the αδ site. In the present study, α-conotoxin SIA also displayed strong affinity for the αγ site but no measurable affinity for the αδ site, thus showing even greater site-selectivity. In contrast, α-conotoxin SI does not distinguish between the two agonist sites, although its sequence differs from that of GI at only three positions: GI, ECCNPACGRHYSC; SI, ICCNPACGPKYSC. Analogues of SI and GI modified at these three positions were studied to identify the determinants of GI's αγ selectivity. Substituting arginine for proline at position 9 produced peptides which displayed "GI-like" selectivity for the αγ site. Conversely, substituting proline for arginine at position 9 resulted in "SI-like" nonselective inhibitors. An SI analogue having alanine in place of proline 9 did not distinguish between the two agonist sites and displayed about the same affinity as SI, indicating the importance of the arginyl cation. Interchanging the residues at position 1 or at position 10 influenced the affinity for the receptor but did not measurably change peptide selectivity. Therefore, of the three sequence differences in SI and GI, the variation at position 9, proline and arginine, respectively, is sufficient to account for GI's selective high-affinity binding to the αγ site on the electric organ acetylcholine receptor.

The nicotinic AChR's[1] from electric organ and embryonic mouse skeletal muscle are pentameric proteins with a subunit stoichiometry of $\alpha_2\beta\gamma\delta$. Although the two agonist-binding sites on each receptor are formed mainly by aromatic residues on the extracellular segments of the two identical α subunits [for review, see (*1*)], the neighboring γ and δ subunits also contribute to agonist site structure (*2*−*5*). It is for this reason that these two sites are not identical but display two different affinities toward agonists and certain competitive antagonists, including the bis-cationic alkaloids such as TC[1] and its analogues (*6*−*8*). The two cationic centers of the alkaloids bridge the subunit interfaces and bind to the αγ site with much higher affinity than to the αδ site on both electric organ and mouse muscle AChR (*9*).

The α-conotoxins are short peptides found in cone snail venoms which also act as competitive antagonists of the AChR (*10*). Like TC, α-conotoxins MI and GI bind to the αγ site on electric organ AChR with much higher affinity than to the αδ site (*11*−*13*). This curare-like selectivity is consistent with a previously-proposed model for α-conotoxin GI binding (*14*), which was based on the earlier model proposed for the bis-cationic alkaloids (*15*). This model proposed that the two cationic centers of GI, the N-terminal amino group and the 9-arginylguanidinium group, interact directly with the AChR. Structural studies on GI have supported this model (*16*−*18*). This model of α-conotoxin binding also agrees generally with structure−function studies on GI and MI analogues (*14*, *19*, *20*). However, the analogy between the bis-cationic alkaloids and the α-conotoxins is not as straightforward as first suggested. α-Conotoxins MI, GI, and SIA all displayed selective high-affinity binding to the αδ (TC low-affinity) site on cell-expressed mouse muscle AChR (*13*, *21*). Therefore, at least in the case of mouse muscle AChR, the α-conotoxin and bis-cationic alkaloid sites are clearly not identical. Indeed, the three residues which determine MI's high-affinity binding to the mouse αδ site (*22*) are distinct from the three residues which determine alkaloid high-affinity binding to the mouse αγ site (*23*).

In contrast to GI and MI, α-conotoxin SI does not distinguish between the two agonist sites on the electric organ AChR, but displays a single, relatively low affinity for both sites (*11*). At first glance, it is somewhat surprising that SI is not selective, since its sequence differs from that of GI at only three positions: the N-terminal residue and midchain residues 9 and 10:

GI: ECCNPACGRHYSC

SI: ICCNPACGPKYSC

These two peptides share the same 2−7 and 3−13 cystine bridges and C-terminal amidation and have been reported to possess similar three-dimensional structures (*30*). The observed difference in agonist-site selectivity between GI and SI must result from one or more of these three sequence differences. In an attempt to identify the peptide molecular basis for this difference in their binding behaviors toward

† This research was supported by grants NIH-RCMI-2G12RR03035 and NIH-MBRS-SO6GM50695. L.M.G. and T.J. are student researchers in the NIH−Minority Biomedical Research Support program.

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⊗ Abstract published in *Advance ACS Abstracts,* July 1, 1997.

[1] Abbreviations: AChR, nicotinic acetylcholine receptor; TC, *d*-tubocurarine; BTX, α-bungarotoxin; ^{125}I-BTX, [^{125}I]Tyr^{54}-α-bungarotoxin.

S0006-2960(97)00807-6 CCC: $14.00

the electric organ AChR, synthetic analogues of SI and GI modified at these three positions, as well as the naturally occurring analogue SIA, were studied.

EXPERIMENTAL PROCEDURES

Materials. Synthetic α-conotoxins SI, GI, and MI (97−99% pure by HPLC), TC, and unlabeled BTX[1] were from Sigma Chemicals. Synthetic α-conotoxin SIA (>97% pure) was from Bachem. ^{125}I-BTX[1] was from DuPont−New England Nuclear. Frozen *Torpedo californica* electric organ was from Pacific Biomarine Lab. Synthetic α-conotoxin SI and GI analogues were made by Biosynthesis Inc. Linear peptides blocked at the cysteines were purified to >95% purity by HPLC and their identities confirmed by mass spectrometry. Cysteines were then selectively cleaved and allowed to oxidize in the presence of potassium ferricyanide to assure formation of cysteine cross-linkings at C2−C7 and C3−C13. Peptides were then repurified on HPLC to >90% purity, and their identities were confirmed by mass spectrometry. Two peptides, SI(P9R) and GI(R9P), could not be successfully synthesized by the above method despite repeated attempts. In these two cases, linear peptides were synthesized without cysteine blocking by Chiron Mimotopes, HPLC-purified to 95% purity, and identified by mass spectrometry. The linear peptide was then allowed to oxidize. In the case of α-conotoxin GI, this has been reported to give greater than 50% yield of the correctly cross-linked peptide (C2−C7; C3−C13) (*24*, *32*). In the cases of SI(P9R) and GI(R9P), HPLC and mass spectrometric analysis of the oxidized mixture revealed that in both cases one major product accounted for about 75% of the peptide present. This mixture was tested without further purification. The total peptide concentration in conotoxin stock solutions was determined by the method of Lowry et al. (*25*) using an α-conotoxin MI standard curve.

Preparation and Characterization of AChR-Rich Membranes. AChR-rich membranes were prepared from the total membrane fraction of homogenized electric organ by ultracentrifugation on a discontinuous sucrose gradient as previously described (*26*). Protease inhibitors were present at all times during the preparation of membranes. The AChR-rich membranes were maintained in buffer A (50 mM NaCl, 10 mM $NaHPO_4$, and 1 mM EDTA at pH 7.4). Total membrane protein concentration was determined by the Lowry method (*25*) using bovine serum albumin as standard. Total AChR was determined from ^{125}I-BTX saturation curves as described previously (*11*). The specific activity of the AChR-rich membranes ranged from 0.5 to 1.5 nmol of ^{125}I-BTX-binding sites (0.25−0.75 nmol AChR) per milligram of protein.

^{125}I-BTX-Binding Assay. In a modification of the method of Schmidt and Raftery (*27*), membranes were diluted 1:200 (v/v) in solubilizing buffer B [buffer A plus 0.1% (v/v) Triton X-100 at pH 7.4] and preincubated for 30 min at room temperature with or without inhibitors. ^{125}I-BTX in buffer B was then added to a final toxin concentration of 10 nM and a final receptor concentration of 2−3 nM, and the samples were incubated for 60 min. Triplicate 50 μL aliquots of each sample were vacuum-filtered through 3-fold 1.3 cm (diethylaminoethyl)cellulose disks (Whatman) prewashed with buffer B. The filters were then quickly washed with 1 mL of buffer B and counted in a Beckman 5500 gamma counter. The amount of specifically bound ^{125}I-BTX was calculated by subtracting the counts in control samples preincubated with 0.6 μM unlabeled BTX. Under these conditions, ^{125}I-BTX binding reached a maximum within 20−30 min, did not change over a 24-h period of incubation, and did not display selectivity or cooperativity within the sensitivity of this assay.

Data Analysis. Binding data normalized to control (absence of inhibitor) were fit to the equation for mutually exclusive ligand displacement from two equimolar independent sites by two inhibitors (*28*):

$$RT/RT_c = [0.5/(1 + I/K_{1I} + J/K_{1J})] + [0.5/(1 + I/K_{2I} + J/K_{2J})] \quad (1a)$$

where RT and RT_c are the concentrations of ^{125}I-BTX−AChR complex in the presence and absence, respectively, of inhibitors, I and J are the inhibitor concentrations, and K_1 and K_2 are the apparent dissociation constants for each inhibitor at each site (inhibitor concentration producing 50% inhibition). In the presence of only one inhibitor ($J = 0$), the equation reduces to

$$RT/RT_c = [0.5/(1 + I/K_{1I})] + [0.5/(1 + I/K_{2I})] \quad (1b)$$

The Hill coefficient (n_H) for total inhibition of ^{125}I-BTX binding was generated by fitting normalized experimental data between 10% and 90% inhibition to the linear Hill equation (*28*):

$$\log [(RT_c/RT) - 1] = n_H \log I + n_H \log IC_{50} \quad (2)$$

where IC_{50} is the inhibitor concentration producing 50% total inhibition.

Model-fitting was performed on data pooled from a minimum of three experiments on PSI-Plot software (Poly Software International) employing the Marquardt−Levenburg nonlinear curve-fitting method. Goodness-of-fit was measured with the coefficient of determination as previously described (*11*).

RESULTS

Interaction of α-Conotoxin SIA with AChR from Electric Organ. α-Conotoxin SIA produced only 50% inhibition of ^{125}I-BTX binding at the maximal peptide concentration of 260 μM (Figure 1). This contrasts with the nonselective inhibitor, SI, and the selective inhibitors, GI and MI, which fully inhibited ^{125}I-BTX binding at around 100 μM concentration under the same conditions. The Hill coefficient for SIA inhibition was 0.76, indicating that it was binding to a fairly homogeneous population of sites. Fitting the data to eq 1b generated an apparent dissociation constant of 586 nM for the detectable binding site of SIA.

These findings suggest that SIA was binding with measurable affinity to only one of the two agonist sites. Inhibition of ^{125}I-BTX binding by TC was measured in the presence of SIA to verify this and to identify the site with which SIA was interacting. In the presence of 8 μM SIA, a concentration sufficient to saturate most of the available SIA-binding sites, TC at concentrations below 1 μM no longer inhibited ^{125}I-BTX binding, while at higher TC concentrations the data

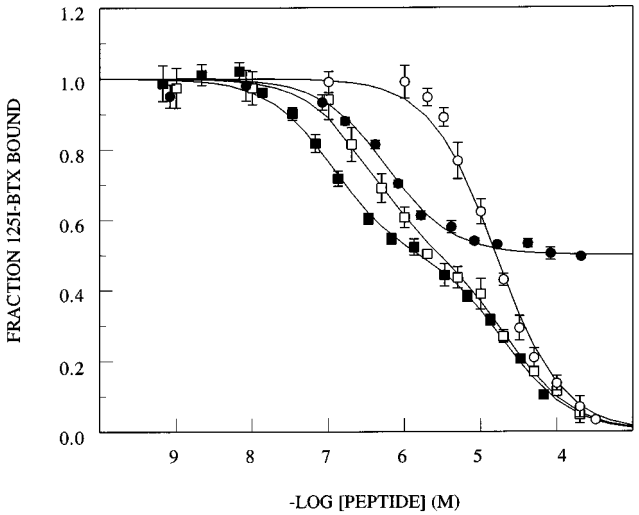

FIGURE 1: Inhibition of ^{125}I-BTX binding to AChR by α-conotoxins SIA, MI, GI and SI. *Torpedo californica* electric organ AChR was detergent-solubilized to a final concentration of 2−3 nM in BTX-binding sites and incubated for 60 min at room temperature with 10 nM ^{125}I-BTX following 30 min preincubation in the presence of SIA (filled circles), MI (filled squares), GI (open squares), or SI (open circles) at the indicated final concentrations. Nonspecific ^{125}I-BTX binding measured in the presence of 0.6 μM unlabeled BTX was subtracted. Each point represents the mean ± 1 standard deviation from at least 3 experiments. Curves were generated by fitting data to the equation for mutually exclusive ligand displacement of two equimolar, independent sites (eq 1b).

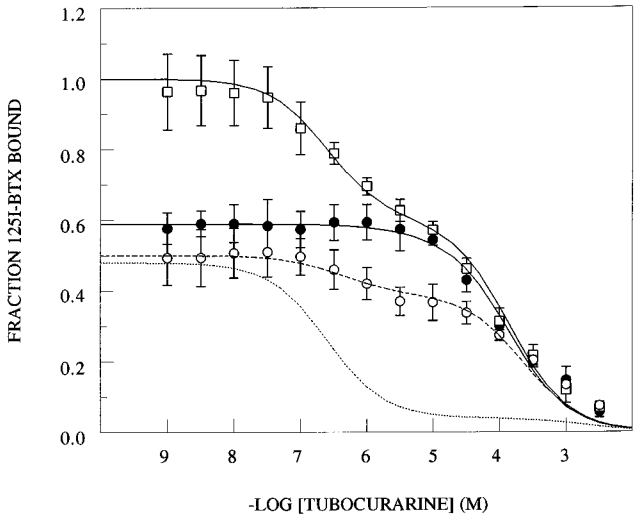

FIGURE 2: Effect of α-conotoxins SIA and SI on inhibition of ^{125}I-BTX binding to AChR by TC. Experimental conditions were as described in Figure 1 except that binding at the indicated concentrations of TC was measured in the absence (open squares) or presence of 8 μM SIA (filled circles) or 10 μM SI (open circles). Each point shows the mean ± 1 standard deviation from 3 experiments. Control data (TC alone) were fit to eq 1b to determine the K_I's for TC (256 nM and 143 μM). Using these parameters, data in the presence of SIA were fit to eq 1a with the single K_I for SIA (586 nM) assigned to coincide either with the TC high-affinity site (solid line) or with the TC low-affinity site (dotted line). In the case of SI, the single K_I (16.1 μM) was assigned to both TC sites (dashed line).

followed the control data obtained in the absence of SIA (Figure 2). The Hill coefficient for TC increased from 0.38 in the control to 0.67 in the presence of SIA, while the IC_{50} for TC increased from 10 μM to 135 μM. These data indicate that SIA was competing with TC mainly at the TC high-affinity site. The TC binding data in the presence of SIA fit well to the model for competitive inhibition in the presence of two inhibitors (eq 1a), but only if the SIA-binding site were assigned to the high-affinity TC site. If the SIA site were assigned to the low-affinity TC site, the data did not fit the model.

If SIA were not binding selectively, the TC inhibition curve in the presence of SIA would be expected to mirror the entire control curve, displaying the same Hill coefficient. This was seen, for example, when TC binding was measured in the presence of the nonselective inhibitor, SI. In this case, the data displayed a Hill coefficient of 0.38, identical to that of the control, and mirrored the control curve (Figure 2).

Therefore, SIA displays a very high degree of site-selectivity toward the electric organ AChR, binding appreciably only to the TC high-affinity ($\alpha\gamma$) site under the conditions used.

Interaction of α-Conotoxin SI and GI Analogues with AChR from Electric Organ. The sequences and binding parameters of the six synthetic α-conotoxin analogues used in this study are shown in Table 1. All six analogues were fully soluble in buffer B at 1.3 mM concentrations, which was the maximum attempted. All six analogues were pharmacologically active in inhibiting ^{125}I-BTX binding to both sites on the solubilized AChR.

Substituting alanine for proline 9 of SI resulted in a peptide, SI(P9A), which continued to display homogeneous binding to both sites on the AChR with a Hill coefficient of 1.12 (Figure 3). Fitting the data to eq 1b generated apparent dissociation constants for the two sites which were identical to each other and similar to those for SI (Table 1). Therefore, SI(P9A) was a nonselective ligand, much like its parent compound.

In contrast, substituting proline 9 of SI with arginine, the residue at position 9 of GI, produced a peptide, SI(P9R), which displayed heterogeneous binding to the AChR (Figure 3). Its Hill coefficient of 0.47 was similar to that of both GI and MI, indicating selective binding (Table 1). The data fit well to eq 1b and generated high- and low-affinity constants whose ratio of 135 was between the ratios of GI and MI.

Conversely, substituting proline for arginine 9 of GI produced a peptide, GI(R9P), which was nonselective, like SI, binding homogeneously with a Hill coefficient of 1.11 (Figure 3). This substitution eliminated GI's selective high affinity for the $\alpha\gamma$ site.

To test whether SI(P9R) displayed the same $\alpha\gamma$ site-specificity as TC and the selective α-conotoxins, GI, MI, and SIA, the inhibition of ^{125}I-BTX binding by TC was measured in the presence of SI(P9R). In the absence of TC, SI(P9R) at 112 nM inhibited ^{125}I-BTX binding by 34%, consistent with the SI(P9R) data shown in Figure 3. With SI(P9R) present at this concentration, TC appeared to be an ineffective competitive inhibitor at low concentrations in comparison with what was observed in the absence of SI-(P9R) (Figure 4). At higher TC concentrations, the inhibition curve approached the shape expected for binding to a single low-affinity site, with the Hill coefficient for TC binding increasing toward 1 (from 0.36 to 0.63) and the IC_{50} for TC increasing from 17 μM to 195 μM. The data fit well to the model for competitive inhibition in the presence of two inhibitors (eq 1a) if the high-affinity SI(P9R) site and the high-affinity TC site were assigned to correspond but not if the opposite assumption were made. Therefore, SI(P9R) binds with selective high affinity to the electric organ AChR

Table 1: Sequences and Binding Parameters of α-Conotoxins and Analogues[a]

peptide	sequence[b]	n_H	K_{I1} (M)	K_{I2} (M)	K_{I2}/K_{I1}
SI	ICCNPACGPKYSC	1.06 ± 0.04	$(1.6 \pm 0.1) \times 10^{-5}$	$(1.6 \pm 0.1) \times 10^{-5}$	1
GI	ECCNPACGRHYSC	0.53 ± 0.02	$(3.6 \pm 0.4) \times 10^{-7}$	$(2.4 \pm 0.2) \times 10^{-5}$	67
SI(P9A)	ICCNPACGAKYSC	1.12 ± 0.04	$(1.3 \pm 0.1) \times 10^{-5}$	$(1.3 \pm 0.1) \times 10^{-5}$	1
SI(P9R)	ICCNPACG**R**KYSC	0.47 ± 0.02	$(2.6 \pm 0.2) \times 10^{-8}$	$(3.5 \pm 0.3) \times 10^{-6}$	135
GI(R9P)	ECCNPACG**P**HYSC	1.11 ± 0.03	$(1.2 \pm 0.1) \times 10^{-4}$	$(1.2 \pm 0.1) \times 10^{-4}$	1
SI(I1E)	**E**CCNPACGPKYSC	1.05 ± 0.09	$(2.2 \pm 0.2) \times 10^{-4}$	$(2.2 \pm 0.2) \times 10^{-4}$	1
GI(E1I)	**I**CCNPACGRHYSC	0.62 ± 0.04	$(3.7 \pm 0.4) \times 10^{-7}$	$(1.3 \pm 0.2) \times 10^{-5}$	35
SI(K10H)	ICCNPACGP**H**YSC	1.18 ± 0.03	$(2.8 \pm 0.2) \times 10^{-5}$	$(2.8 \pm 0.2) \times 10^{-5}$	1
MI	GRCCHPACGKNYSC	0.44 ± 0.02	$(1.2 \pm 0.1) \times 10^{-7}$	$(2.1 \pm 0.1) \times 10^{-5}$	175
SIA	YCCHPACGKNFDC	0.76 ± 0.06[c]	$(5.9 \pm 0.4) \times 10^{-7}$	$> 2.6 \times 10^{-4}$	>440

[a] Inhibition of ^{125}I-BTX binding to detergent-solubilized *Torpedo californica* electric organ AChR was measured as described under Experimental Procedures. The Hill coefficient (n_H) was generated by fitting data between 10% and 90% inhibition to eq 2. Apparent dissociation constants (K_I's) were generated by fitting data to eq 1b. Values shown are means ± 1 standard deviation of the fitting procedure. [b] All peptides have free N-termini, amidated C-termini, and disulfide bridges at C2−C7 and C3−C13 (except MI at C3−C8 and C4−C14). Sequence differences between GI and SI are underlined. Analogue differences from parent α-conotoxins are in boldface type. [c] Single binding site only. Peptide achieved only 50% total inhibition at the highest concentration studied (260 μM).

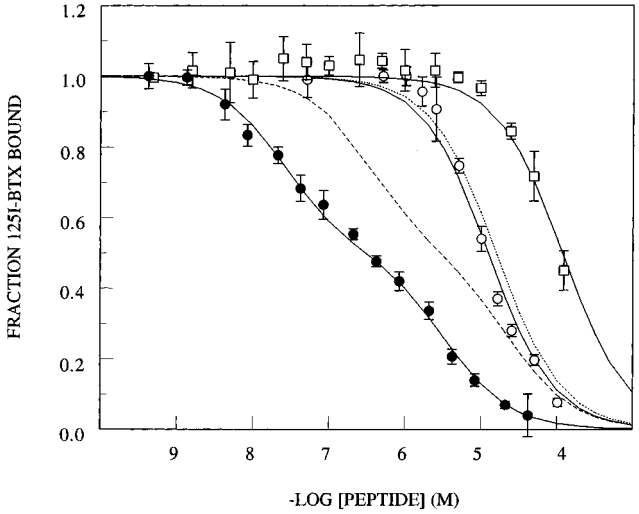

FIGURE 3: Inhibition of ^{125}I-BTX binding to AChR by α-conotoxin analogues SI(P9A), SI(P9R), and GI(R9P). Experimental conditions and data analysis were as described in Figure 1 except that incubation was in the presence of SI(P9A) (open circles), SI(P9R) (filled circles), or GI(R9P) (open squares) at the concentrations indicated. The inhibition curves from Figure 1 for SI (dotted line) and GI (dashed line) are also shown.

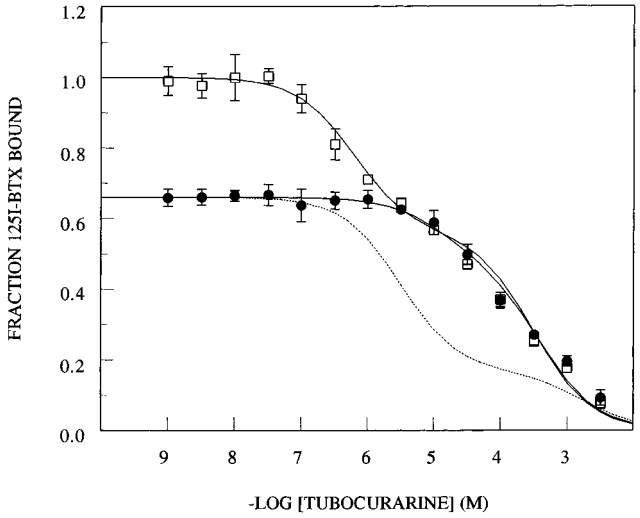

FIGURE 4: Effect of the α-conotoxin analogue SI(P9R) on inhibition of ^{125}I-BTX binding to AChR by TC. Experimental conditions were as described in Figure 1 except that binding at the indicated concentration of TC was measured in the absence (open squares) or presence (filled circles) of 112 nM SI(P9R). Each point shows the mean ± 1 standard deviation from 3 experiments. Control data for TC alone were fit to eq 1b to determine the K_I's for TC (628 nM and 316 μM). Using these parameters and the K_I's for SI(P9R) (26 nM and 3.5 μM), data in the presence of SI(P9R) were fit to eq 1a with the TC and SI(P9R) high-affinity sites assigned either to coincide (solid line) or not to coincide (dotted line).

αγ site, like the other selective competitive antagonists, TC and α-conotoxins GI, MI, and SIA.

Interchanging residues at the N-terminal (position 1) did not alter the peptide's binding site behavior from that of the parent peptide. Analogue SI(I1E) continued to display homogeneous binding with a Hill coefficient of 1.05 while analogue GI(E1I) continued to display heterogeneous binding with a Hill coefficient of 0.62 (Figure 5). Fitting the SI(I1E) data to eq 1b generated a single apparent dissociation constant while fitting the GI(E1I) data generated high- and low-affinity constants with a 35-fold difference (Table 1). TC inhibition studies in the presence of GI(E1I) showed that the peptide's high-affinity site corresponds to the αγ site (data not shown).

Substituting the lysine at position 10 of SI with histidine, the residue at position 10 of GI, did not introduce site-selectivity. Analogue SI(K10H) continued to display homogeneous binding (Figure 5) with a Hill coefficient of 1.18 and a single affinity constant on fitting the data to eq 1b. The corresponding GI position 10 substitute, GI(H10K), was not synthesized.

DISCUSSION

α-Conotoxin SIA Is a Highly Selective Ligand for the αγ Site on Electric Organ AChR. α-Conotoxin SIA showed fairly high affinity for the αγ site (K_I = 590 nM), but no measurable affinity for the αδ site up to a peptide concentration of 260 μM. Therefore, SIA displayed a high degree of site-selectivity (K_{I2}/K_{I1} > 440) for the electric organ AChR. SIA also showed marked selectivity for the mouse AChR αδ site, but binding to its mouse low-affinity (αγ) site was still within measurable limits (IC_{50} = 200 μM) (*13*). Therefore, SIA is even more specific for the electric organ AChR αγ site than for the mouse αδ site and may prove to be a particularly valuable tool in probing the electric organ AChR agonist-binding domains.

Since α-conotoxin MI differs from SIA at only three positions (Table 1) and yet shows much higher affinity for the electric organ αδ site, studies using synthetic MI and SIA analogues, similar to those reported here on GI and SI

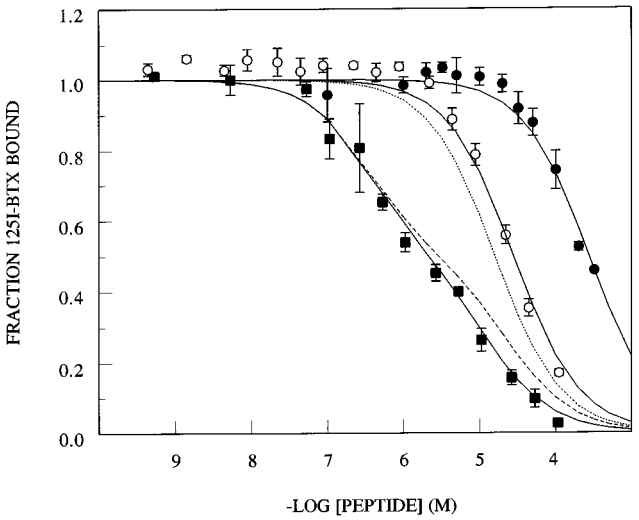

FIGURE 5: Inhibition of ^{125}I-BTX binding to AChR by analogues SI(I1E), GI(E1I), and SI(K10H). Experimental conditions and data analysis were as described in Figure 1 except that the peptides present were SI(I1E) (filled circles), GI(E1I) (filled squares), or SI(K10H) (open circles). Inhibition curves from Figure 1 for SI (dotted line) and GI (dashed line) are also shown.

analogues, could be performed to identify the determinants of SIA's extreme site-selectivity and shed further light on how the α-conotoxins interact with the AChR.

Of the Three Sequence Differences in SI and GI, the Variation at Position 9, Proline and Arginine, Respectively, Is Sufficient To Account for GI's Selective High-Affinity Binding to the αγ Site on the Electric Organ AChR. The findings in this report show that an arginine residue at position 9 on the α-conotoxin scaffold confers selective high-affinity toward the αγ site. This was seen with three different peptide pairs, SI/SI(P9R), GI(R9P)/GI, and SI-(K10H)/GI(E1I), where the only difference is proline or arginine, respectively, at position 9. In all three cases, the 9-proline peptide binds nonselectively while the 9-arginine peptide binds with selectively higher affinity to the αγ site. Simply replacing proline 9 of SI with a neutral residue, alanine, did not produce selective high-affinity binding, indicating that the rotational restriction around α-carbon 9 imposed by proline is not, by itself, the cause of SI's lack of selectivity. Therefore, the cationic group of arginine probably plays a major role in GI's high affinity for the αγ site.

The findings also indicate that the differences at residues 1 and 10 of SI and GI do not contribute to GI's selectivity for the αγ site. With all three pairs of peptides whose partners differ only in having isoleucine or glutamate at position 1, the partners displayed the same binding behavior. SI and SI(I1E) both bound nonselectively, as did SI(K10H) and GI(R9P), while GI(E1I) and GI both displayed selective high affinity toward the αγ site. In other words, both isoleucine and glutamate at position 1 are compatible with either nonselective or selective binding. Along this same line of reasoning, with all three pairs of peptides whose partners differ from each other only in having lysine or histidine at position 10, again the partners displayed the same binding behavior. SI and SI(K10H) both bound nonselectively, as did SI(I1E) and GI(R9P), while SI(P9R) and GI-(E1I) both bound selectively. Both lysine and histidine at position 10 are also compatible with either nonselective or selective binding.

Therefore, the 9-arginine residue of α-conotoxin GI is responsible for the peptide's selective high affinity toward the αγ site on the electric organ AChR.

These results are consistent with the observation that two other electric organ AChR αγ-selective α-conotoxins, MI and SIA, also have a cationic residue, in this case lysine, at the homologous position of arginine 9 of GI (Table 1). Substituting lysine 10 with either proline or histidine on cloned analogues of MI also resulted in nonselective peptides (*29*). It was also reported that replacing proline 9 of SI with lysine confers GI-like selectivity (*31*). Therefore, a midchain cationic residue immediately distal to the highly conserved CC(N/H)PACG segment appears to be a generally important determinant of α-conotoxin αγ site-selectivity on the electric organ AChR.

Observing the proline-to-arginine-induced selectivity in three different peptide pairs, together with the nonselectivity of SI(P9A), provides good evidence that arginine 9 of GI is in direct contact with the AChR through its cationic group, as previously suggested (*14*, *16*, *17*). This residue in GI is located at the point of a sharp turn in the peptide chain which is stabilized by hydrogen bonding (*16*−*18*). Since the affinity changes were observed at the αγ site and since the two α subunits have identical sequences, the cationic group of arginine 9 probably interacts with one or more nucleophilic residues on the γ subunit.

Because of the similarity of the AChR subunit scaffolds, arginine 9 probably also associates, although more weakly, with the homologous domain on the δ subunit. Evidence for this can be seen by comparing the apparent dissociation constants of three nonselective peptides, SI, SI(K10H), and GI(R9P), with the low-affinity constants (K_{I2}'s) of the corresponding analogues in which proline 9 is substituted with arginine, SI(P9R), GI(E1I), and GI (Table 1). These low-affinity constants represent peptide affinity for the αδ site. In all three cases, the arginine 9 analogue displays 2−5-fold higher affinity for the αδ site. Again, this is not due to the introduction of free rotation around α-carbon 9, since SI and SI(P9A) show about the same affinity for the αδ site.

α-Conotoxin Residues at Positions 1 and 10 Also Influence Binding. Although the sequence difference between SI and GI at position 1 does not contribute to GI's selective high affinity for the αγ site, this residue does appear to influence low-affinity peptide binding to the AChR. In all three pairs of peptides whose partners differ only in having isoleucine or glutamate, respectively, at position 1, the isoleucine peptide shows higher affinity for the low-affinity sites. In the case of the two pairs of nonselective peptides, SI and SI(I1E) and SI(K10H) and GI(R9P), this effect is, of course, seen at both the αγ and αδ sites and ranges from 4- to 13-fold (see Table 1). These findings are consistent with interaction of the N-terminal cation adjacent to residue 1 with two equivalent domains on the AChR as was previously suggested by others (*14*, *16*, *17*). In the case of the pair of selective peptides having arginine at position 9, GI(E1I) and GI, the isoleucine effect is seen only at the low-affinity (αδ) site. However, this effect is less than 2-fold, probably because the adjacent arginine 9 also influences binding to the αδ site, as noted above.

Comparing the three pairs of peptides SI/SI(K10H), SI-(I1E)/GI(R9P), and SI(P9R)/GI(E1I), whose partners differ only in having lysine or histidine, respectively, at position 10, indicates that this difference has little, if any, effect on

low-affinity binding. However, the selective peptide, SI-(P9R), displayed greater than 10-fold higher affinity for the $\alpha\gamma$ site than GI(E1I) or (GI), suggesting that a strong second cation adjacent to arginine 9 facilitated binding to that site.

A Refined Model for α*-Conotoxin Binding to the AChR.* Overall, the findings with synthetic α-conotoxin SI and GI analogues are consistent with the original model for α-conotoxin GI binding to the AChR (*14*). The data suggest refinement of that model, in the case of the electric organ AChR, where the residue 9-cation binds to the γ subunit strongly and to the δ subunit weakly, while the N-terminal cation binds to apparently equivalent electronegative domains at the $\alpha\gamma$ and $\alpha\delta$ sites. This N-terminal cation subsite may well be the agonist cation-binding site formed mainly by aromatic α subunit residues (*1*), but further studies are needed to confirm this hypothesis.

While the α-conotoxin high-affinity site is $\alpha\gamma$ on the electric organ AChR, it is $\alpha\delta$ on the mouse muscle AChR (*13*, *21*). The determinants of α-conotoxin MI's high affinity for the mouse $\alpha\delta$ site are residue pairs γK34/δS36, γS111/δY113, and γF172/δI178 (*22*). The γS111/δY113 difference appears to be especially important for MI's high affinity and suggests an important cation−tyrosine interaction at the high-affinity α-conotoxin site. Although the residues responsible for α-conotoxin high affinity toward the electric organ AChR $\alpha\gamma$ site have not yet been identified, the subunit sequences of *Torpedo californica* electric organ and mouse muscle AChR's are either identical or very similar at positions γ34, δ36, γ172, and δ178:

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                    34              111             172
  Mouse γ   ...V-S-L-K-L-T-L...V-L-V-S-P-D-G...E-W-I-F-I-D-P...
Torpedo γ   ...V-T-L-K-L-T-L...V-L-V-Y-N-D-G...E-W-I-H-I-D-P...

                    36              113             178
  Mouse δ   ...V-A-L-S-L-T-L...V-L-V-Y-D-S-G...E-W-I-I-I-D-P...
Torpedo δ   ...I-A-L-S-L-T-L...V-L-V-R-P-N-G...E-W-I-I-I-D-P...
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However, the electric organ AChR γ subunit has tyrosine in place of serine at position 111, while its δ subunit has arginine in place of tyrosine at position 113. These two differences may account for the α-conotoxin site-specificity difference between these two species. In the case of GI, the putative cation−tyrosine interaction could theoretically involve either the N-terminal amino group or the 9-arginylguanidinium group. The present studies suggest that it is the 9-arginylguanidinium group of the peptide which interacts with tyrosine 113 on the mouse δ subunit and, by analogy, with tyrosine 111 on the electric organ γ subunit.

ACKNOWLEDGMENT

We thank Milagros Hernandez and Andrés Vergara for skillful technical assistance.

REFERENCES

1. Karlin, A. (1993) *Curr. Opin. Neurobiol. 3*, 299−309.
2. Blount, P., and Merlie, J. P. (1989) *Neuron 3*, 349−357.
3. Pedersen, S. E., and Cohen, J. P. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 2785−2789.
4. Sine, S. M., and Claudio, T. (1991) *J. Biol. Chem. 266*, 19369−19377.
5. Unwin, N. (1996) *J. Mol. Biol. 257*, 586−596.
6. Damle, V. K., and Karlin, A. (1978) *Biochemistry 17*, 2039−2045.
7. Neubig, R. R., and Cohen, J. B. (1979) *Biochemistry 18*, 5464−5475.
8. Sine, S. M., and Taylor, P. (1981) *J. Biol. Chem. 256*, 6692−6699.
9. Fu, D.-X., and Sine, S. M. (1994) *J. Biol. Chem. 269*, 26152−26157.
10. Gray, W. R., Olivera, B. M., and Cruz, L. J. (1988) *Annu. Rev. Biochem. 57*, 665−700.
11. Hann, R. M., Pagán, O. R., and Eterović, V. A. (1994) *Biochemistry 33*, 14058−14063.
12. Utkin, Y. N., Kobayashi, Y., Hucho, F., and Tsetlin, V. I. (1994) *Toxicon 32*, 1153−1157.
13. Groebe, D. R., Dumm, J. M., Levitan, E. S., and Abramson, S. N. (1995) *Mol. Pharmacol. 48*, 105−111.
14. Gray, W. R., Middlemas, D. M., Zeikus, R., Olivera, B. M., and Cruz, L. J. (1985) in *Peptides, Structure and Function. Proceedings of the 9th American Peptide Symposium* (Deber, C. M., Hruby, V. J., and Kopple, K. D., Eds.) pp 823−832, Pierce Chemical Co., Rockford, IL.
15. Pauling, P., and Petcher, T. J. (1973) *Chem.-Biol. Interactions 6*, 351−356.
16. Kobayashi, Y., Ohkubo, T., Kyogoku, Y., Nishiuchi, Y., Sakakibara, S., Braun, W., and Go, N. (1989) *Biochemistry 28*, 4853−4860.
17. Pardi, A., Galdes, A., Florance, J., and Maniconte, D. (1989) *Biochemistry 28*, 5494−5501.
18. Guddat, L. W., Martin, J. A., Shan, L., Edmundson, A. B., and Gray, W. R. (1996) *Biochemistry 35*, 11329−11335.
19. Hashimoto, K., Uchida, S., Yoshida, H., Nishiuchi, Y., Sakakibara, S., and Yukari, K. (1985) *Eur. J. Pharmacol. 118*, 351−354.
20. Almquist, R. G., Kadambi, S. R., Yasuda, D. M., Weitl, F. L., Polgar, W. E. and Toll, L. R. (1989) *Int. J. Pept. Protein Res. 34*, 455−462.
21. Kreienkamp, H.-J., Sine, S. M., Maeda, R. K., and Taylor, P. (1994) *J. Biol. Chem. 269*, 8108−8114.
22. Sine, S. M., Kreienkamp, H.-J., Bren, N., Maeda, R., and Taylor, P. (1995) *Neuron 15*, 205−211.
23. Sine, S. M. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 9436−9440.
24. Nishiuchi, Y., and Sakakibara, S. (1982) *FEBS Lett. 148*, 260−262.
25. Lowry, O. H., Roseborough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem. 193*, 265−275.
26. Szczawinska, K., Ferchmin, P. A., Hann, R. M., and Eterović, V. A. (1992) *Cell. Mol. Neurobiol. 12*, 95−106.
27. Schmidt, J., and Raftery, M. A. (1973) *Anal. Biochem. 52*, 349−354.
28. McGonigle, P. W., and Molinoff, P. B. (1994) in *Basic Neurochemistry* (Siegle, G. J., Agranoff, B. W., Albers, R. W., and Molinoff, P. B., Eds.) 5th ed., pp 209−230, Raven Press, New York.
29. Ulloa, J. S., Sanchez, M. V., Baksi, K., Pagán, O. R., and Hann, R. (1995) *Abstracts of the 5th International Symposium on Neurotoxins in Neurobiology*, p 7.
30. Mitchell, S. S., Shon, K. J., Olivera, B., and Ireland, C. M. (1996) *J. Nat. Toxins 5*, 191−208.
31. Groebe, D. R., Gray, W. R., and Abramson, S. N. (1997) *Biochemistry 36*, 6469−6474.
32. Zhang, R., and Snyder, G. H. (1991) *Biochemistry 30*, 11343−11348.

BI970807C